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Evaluation of hemostatic activity of latex from three *Euphorbiaceae* species

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ABSTRACT

Ethnopharmacological relevance: Latices from several plant species of *Euphorbiaceae* family have been traditionally applied over fresh cuts to stop bleeding and subsequently applied over wounds to enhance healing process. The latex arrested bleeding from fresh wounds by reducing bleeding and whole blood coagulation time which are important indices of hemostatic activity. It has been accepted that hemostatic activity is due to the proteolytic fraction of plant latices. Thus, the present study aimed to assess the clot inducing properties of three *Euphorbiaceae* plants viz., *Euphorbia nivulia* Buch.-Ham., *Pedilanthus tithymaloides* (L.) Poit and *Synadenium grantii* Hook F.

Materials and methods: In the present study, various proteolytic activities namely protease, gelatinase, milk clotting and whole blood clotting assay of the enzyme fraction of latices of *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii* have been investigated. The inhibition profile of protease specific inhibitors was assessed. Also, the effects of protein fractions were studied using bleeding/clotting time test of fresh experimentally-induced wounds in mice.

Results: *Euphorbia nivulia* latex protease has noticeable blood clotting activity followed by *Pedilanthus tithymaloides* and *Synadenium grantii*. Stem latex protease of *Pedilanthus tithymaloides* exhibits superior procoagulant activity in different mammal's blood samples viz., *Capra hircus*, *Bubalus bubalis*, *Ovibos moschatus* and *Bos indicus*. Blood sample of ox was the most sensitive to latex protease than other mammal's blood. Concomitantly, the plant latex protease could significantly reduce whole blood clotting time of human and mice blood samples.

Conclusion: The protease fraction of latices of *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii* possesses phytoconstituents capable of arresting wound bleeding, and accelerating whole blood coagulation process. It suggests good potentiality for use of latex proteases in wound management. Also, the finding of this study showed that the protease enzyme of *Pedilanthus tithymaloides* has the most potent hemostatic agent.

1. Introduction

The normal hemostatic mechanism involves normal functions of blood vessels, platelets and the blood coagulation. Blood coagulation is a complex process by which blood forms clots. It is an important part of hemostasis in which a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel (Satish et al., 2012). When the vessel wall is damaged, the subendothelial structures, including basement membrane, collagen and microfibrils, are exposed. The platelets adhere to the exposed damaged endothelium, the outer membrane becomes stickier so that other platelets can adhere to it form platelet aggregates called platelet plug (Oduola et al., 2005). Disorders of coagulation can lead to an increased risk of bleeding (hemorrhage) or clotting (thrombosis). In patients with advanced liver disease, bleeding and thrombosis are dangerous complications, particularly in those who are challenged by infection or who require surgery (Satish et al., 2012).

World Health Organization (WHO) reported that about 80% African, 75% French, 70% Canadian, 42% American and 40% of Chinese population depends on traditional medicine for the completion of their day to day needs regarding primary health (Shivaprasad et al., 2011). Since 1978, WHO has developed a global classification of traditional medicine, therefore the biological activities of various plant extracts have been demonstrated. Till today more than 170,000 potent phytomolecules have been characterized from plant (Victorien et al., 2012). They have proved to be effective in the maintenance and improvement of health

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related problems without causing any adverse side effects. Over centuries researchers are trying to explore the biochemical basis of the remedial effects of phytocompounds or their derivatives. Their findings in course of time ultimately gave rise to pharmacologiclally active molecules. Despite these results, few plants and their medicinally important parts have been studied for their therapeutic properties and few pharmacological activities. Especially, antimicrobial, anti-inflammatory, antidiabetic, hepatoprotective etc activities of plant based extracts have been most popularly studied in India. Few pharmacological activities for example hemostatic effects of plant based extracts remain unexplored.

Accumulated evidences indicate that the latex bearing plants used in the management of various diseases such as diabetes, asthma, dysentery, diarrhea, malaria and skin problems (Badgujar, 2011). Plant latex is the milky juice, found in long branching tubes known as latex tubes. This juice is white, yellow or pinkish in color. It is a viscous fluid and colloidal in nature. Known ingredients of latex are proteins, alkaloids, tannins, terpens, starch, sugars, oils, resins, gums and enzymes (Pandey, 2001). Latices from several plant families viz., Asclepiadaceae species i.e. *Calotropis procera* and *Calotropis gigantea*; *Euphorbiaceae* species i.e. *Jatropha curcas* and *Euphorbia ligularia*; Papaveraceae member i.e. *Argemone mexicana* and Moraceae species such as *Ficus hispida* are applied over injuries or cuts to stop bleeding and also subsequently applied over wound to enhancing healing. So far, only a few plant latices used in folk medicine have been well studied and documented. *Ipomoea carnea* Jacq (Convolvulaceae) and *Euphorbia hirta* (*Euphorbiaceae*) were reported for wound healing activity (Jaiprakash et al., 2006; Ambiga et al., 2007). Curcain is a proteolytic enzyme isolated from latex of *Jatropha curcas*. It has been reported for wound healing activity (Nath and Dutta, 1992). The latex of *Jatropha gossypifolia* was reported for its hemostatic activity (Oduola et al., 2007). The stem latex of *Vilayati sher* is routinely used in Northeastern part of India by herbalists, rural dwellers and some people in urban centers to stop bleeding from nose and skin (Gangwar and Ramakrishnan, 1990). Ethnomedicinally, latices of three members of *Euphorbiaceae* viz., *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii* have been used for stopping the blood from injuries, open cuts or wound and wound healing purposes by indigenous people of not only India but also other nations (Kirtikar and Basu, 1995; Rajesh et al., 2006; Mahajan and Badgujar, 2008; Ghosh et al., 2012).

Phytochemically, *Pedilanthus tithymaloides* was reported for octacosanol, cycloartenone, oxime and beta-sitosterol. The leaves contain *n*-hentriacontanol and dehydrodammaronol-A. The root gave azafrin (Khare, 2007). Pharmacologically, *Pedilanthus tithymaloides* was cited for a wide range of healing properties, namely emetic, anti-inflammatory, antibiotic, antiseptic, antihemorrhagic, antiviral, antitumoral and abortive activity (Abreu et al., 2006). Phytochemical studies of the latex of *Synadenium grantii* yielded a novel skin irritant called 12-O-tigloyl-4-deoxyphorbol-13-isobutyrate (Kinghorn, 1980). Additionally, *Synadenium grantii* has led to the isolation of Four anthocyanins from leaves (Andersen et al., 2010), viz., cyanidin 3-O-(2″-(5‴-(E-p-coumaroyl)-beta-apiofuranosyl)-beta-xylopyranoside)-5-O-beta-glucopyranoside, cyanidin 3-O-(2″-(5‴-(E-p-coumaroyl)-beta-apiofuranosyl)-beta-xylopyranoside), cyanidin 3-O-(2″-(5‴-(E-caffeoyl)-beta-apiofuranosyl)-beta-xylopyranoside) and cyanidin 3-O-(2″-(5‴-(E-feroyl)-beta-apiofuranosyl)-beta-xylopyranoside). Latex of *Synadenium grantii* is widely used in the treatment of various diseases such as allergies, gastric disorders, and especially, in cancer therapy (Ortêncio, 1997). Phytochemical studies of *Euphorbia nivulia* has led to the isolation of ingol diterpenes (3-acetyl-8-methoxyl-7-angolyl-12-hydroxylingol; 3,12-diacetyl-7-hydroxy-8-methoxylingol; 3,12-diacetyl-7-angolyl-8-hydroxylingol; 3,12-diacetyl-8-benzoylingol and 3,12-diacetyl-7-benzoyl-8-nicotinylingol) along with three macrocyclic ingol diterpenes derivatives (3,7,12-triacetyl-8-benzoylingol; 3,12-diacetyl-7-angeloyl-8-methoxyingol and 7-angeloyl-12-acetyl-8-methoxyingol) (Badgujar and Mahajan, 2012). The latex of *Euphorbia nivulia* has been cited for its antioxidant, immunomodulator, cytotoxic, anti-inflammatory, antiproliferative, wound healing, and hemostatic activity (Mahajan and Badgujar, 2011). The pharmacological application of plant Latices as hemostatic and wound healing purpose imply their role on hemostasis and fibrinolysis. Hemostasis and fibrinolysis both are two separate processes maintained by the action of specific protease enzyme/proteins. Therefore, the aim of present study is to investigate the coagulant property of some latices of *Euphorbiaceae* species viz., *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii*, which include the experimental procedures on coagulation of whole blood clotting time of different mammals, which refers to a hemostatic agent, that participate in blood coagulation cascade.

2. Materials and methods

2.1. Plant material and collection of latex

Plant specimen samples of *Euphorbia nivulia* (LAT 87), *Pedilanthus tithymaloides* (LAT 102) and *Synadenium grantii* (LAT 105) were identified from Dr. D.A. Patil, Taxonomist, Department of Botany, North Maharashtra University, Jalgaon, India. Specific voucher specimens of these plants were deposited in the Herbarium of Botany Department, Faculty of Science, M.J. College, Jalgaon, India for future reference. Plant latices of these plants were collected early in the morning by nipping leaves near the stem or by incision of trunk and branches of plant and allowing the milk to drain in clean glass tube separately during rainy season from June 2010 to October 2012, brought to the laboratory and kept in refrigerator (till the experiment starts).

2.2. Animals

Swiss albino mice, domestic goat (*Capra hircus*), buffalo (*Bubalus bubalis*), ox (*Ovibos moschatus*) and cattle (*Bos indicus*) of either sex were used for the present study. The experimental protocol was approved by Institutional Animal Ethics Committee constituted under CPCSEA rules, India (Bioethical allowance number: CPCSEA/IAEC/2010-11/01).

2.3. Preparation of crude enzyme extract

All operations were carried out at 0–5 °C. Latex was homogenized in a homogenizer under chilled condition and filtered through four folds of muslein cloth. Filtrate latex sample was centrifuged at 16,000 ± 100*g* for 45 min at 4 °C. The resulting supernatant of latex fraction is called “crude enzyme extract” or “centrifugal fraction (CF)”, which was used for further investigation of protease enzyme activities.

2.3.1. Protease assay

Proteolytic activity was determined by colorimetric assay using 1% casein (Hammarsten grade) as a substrate as described by Khan et al., 1979. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to μg of tyrosine/min/mg protein at 37 °C and protein content was measured according to Lowry's method using bovine serum albumin as the standard protein (Lowry et al., 1951).

2.3.2. Milk clotting assay

The enzyme was assayed as described by Greenberg method (El-Bendary et al., 2007) with some modification. The time

necessary for the formation of milk clot was measured and its validity was confirmed by using pointed curve needle. Milk clotting activity is expressed in terms of Soxhlet unit.

2.3.3. Gelatinolytic assay

Gelatinolytic activity was determined by colorimetric assay using 0.5% gelatin as substrate as described by Tran and Nagano (2002) and Hamza et al. (2006). The gelatinase activity was expressed as amount of enzyme required to produce peptide equivalent to μmol of leucine/min/mg protein at 37 °C.

2.3.4. Coagulation time of whole blood

Twelve tubes were numbered from T_1 to T_{12}. T_1–T_6 served as control group and T_7–T_{12} acts as test group. T_7 to T_{12} were respectively given 20, 40, 60, 80, 100 and 120 μl of CF fraction of enzyme (latex) and maintain the temperature (37 °C). 500 μl volume of blood were added to each tube, immediately the blood started flowing into the syringe, the tubes were observed for clot formation and the clotting time taken using a stop watch (Victorien et al., 2012).

2.4. Inhibition profile of CF fraction of enzyme

For inhibition profile specific inhibitors of proteases viz., serine protease inhibitor (phenylmethanesulfonyl fluoride i.e. PMSF), aspartic protease inhibitor (pepstatin A), metalloprotease inhibitor (1,10 phenathroline and ethylenediaminetetraacetic acid i.e. EDTA) and cysteine protease inhibitor (iodoacetic acid i.e. IAA and mercuric chloride i.e. $HgCl_2$) were used.

2.4.1. Effect of inhibitors on protease and gelatinase assay

Inhibition of the hydrolysis of casein and gelatin by CF fraction of enzyme was investigated using protease specific inhibitors. The enzyme preparation was incubated with inhibitors individually at 37 °C temperature for 60 min. The residual proteolytic and gelatinolytic activity against casein and gelatin were determined by the standard assay procedure as indicated above. Controls were prepared by preincubating the enzyme fraction with the appropriate solvent used to dissolve the inhibitors. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

2.4.2. Effect of inhibitors on milk clotting and blood clotting activity

To determine the class and specificity of the milk clotting and blood clotting protease, enzyme preparation (CF fraction of enzyme) was incubated at 37 °C temperature for 60 min with the above mentioned inhibitors separately. Then, the residual milk clotting activity against skimmed milk and whole blood clotting profile were measured by the standard assay procedure as indicated above. Controls were prepared by preincubating the enzyme fraction with the appropriate solvent used to dissolve the inhibitors. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken 100%.

2.5. Phytochemical screening

The CF fraction of plant latex was analyzed for their phytochemical composition by qualitative method. Dragendorff's reagent and Mayer's reagent were used to know the presence of alkaloids and cynogenic glycosides using cold concentrated sulfuric acid test. The CF fraction of latex was tested for phenolics using Folin Ciocalteu reagent. Flavonoids were detected by appearance of effervescences with pink color by dissolving CF fraction of latex in 10% HCL and zinc powder. The CF fraction of latex was also tested for terpenoids and saponins. Tannins were detected using gelatin salt block test (Harborne, 1984; Wagner et al., 1984).

2.6. Bleeding time

The effect of the CF fraction of enzyme on bleeding from fresh experimentally induced wounds was evaluated using the bleeding time test in mice (Okoli et al., 2007). After sterilizing the skin with 70% alcohol; a puncture was made on the tail with a sterile sharp blade. Immediately, a drop of the CF fraction of latex (50 and 100 μg protein content) was tropically applied on cut portion and at the same time a stopwatch was switched on. Sterilized filter paper was used to absorb blood coming out and time taken for ceasing bleeding was recorded, the average was taken as bleeding time (test). The procedure was repeated on the second group of animal (mice) but here, after puncturing the tail, a drop of CF fraction of enzyme was not applied, due to it serves as a control group of animal.

2.7. Skin toxicity test

Tests were performed following the guideline of Organisation for Economic Co-operation and Development (OECD) for testing of foreign chemicals. 24 h before the test, animals' furs of dorsal trunk were shaved. This operation was performed with care avoiding skin injury which could alter its permeability. An area of 20 mm × 10 mm was generated. At the beginning of the experiment, mice were placed in individual cages. The mice were randomly divided into two groups of six mice: test group-mice treated with CF fraction and control group-mice treated with normal saline. For each group of mice, CF fraction or normal saline was uniformly applied on a free surface of the skin. It was held in contact with the skin with a porous gauze dressing and non irritating tape for a period of 4 h. At the end of exposure, any residual CF fraction was removed with the help of buffer. Skin reactions at the portion of the treated skin were visually evaluated at 1, 24, 48, 72 h, 7 days and 14 days after treatment. Scores corresponding to the skin reactions were attributed according to the scoring system described by OECD test guidelines.

2.8. Statistical analysis

The mean and standard deviation and the level of significance for the difference between means were determined by Tukey's Multiple Comparison Test (Daniel, 2004) and were computed by GraphPad Prism 4.

3. Results

A complete detail of identified laticiferous plants belongs to *Euphorbiaceae* family with botanical name, voucher specimen number, vernacular name, habitat, nature and part used as a source of latex and their ethnomedicinal application with respect to hemostatic activity is summarized in Table 1. The result in Table 2 highlights the plant latices having proteolytic, milk clotting and gelatinolytic activity. The order of potentiality with respected proteolytic activity of latex proteases are *Pedilanthus tithymaloides* > *Synadenium grantii* > *Euphorbia nivulia* and with respect to milk clotting activity is *Euphorbia nivulia* > *Synadenium grantii* > *Pedilanthus tithymaloides*. On the other hand this order is having different magnitude sequence for gelatinase assay i.e. *Pedilanthus tithymaloides* > *Euphorbia nivulia* > *Synadenium grantii*. It means that stem latex protease of *Pedilanthus tithymaloides* is proteolytically and gelatinolytically the most potent candidate whereas *Euphorbia nivulia* latex protease is having noticeable milk clotting activity.

Table 1
Details of the plants and their parts used in present investigation.

Sr. no.	Botanical name and voucher specimen no.	Vernacular name and part used[a]	Habitat[b] and nature[c]	Period of flowering	Appearance	Traditional uses	Reference
1	*Euphorbia nivulia* Buch.-Ham. (LAT 87)	*Sabar*, SB	S, WD	March–June	Wild on dry and rocky regions	Blood flow from wound is immediately stopped by applying milky juice over the surface of wound	Kirtikar and Basu (1995); Mahajan and Badgujar (2008)
2	*Pedilanthus tithymaloides* (L.) Poit (LAT 102)	*Vilayati sher*, SB, LF	H, OR	March–May	Planted as hedge in garden and around bungalows	Milky juice of leaves and stem bark is applied locally thrice a day on wound, it heals quickly	Ghosh et al. (2012)
3	*Synadenium grantii* Hook F. (LAT 105)	*Shend*, SB	S, OR	March–May	Planted along fences	Milky latex of the plant used to treat on wound healing	Rajesh et al. (2006)

[a] Part used: SB: stem bark and LF: leaf.
[b] Habitat: S: shrub and H: herb.
[c] Nature: WD: wild and OR: ornamental.

Table 2
Proteolytic activities of laticiferous plants.

Sr. no.	Botanical name	Protein (mg/g latex)	PA (U/g latex)	MCA (U/g latex)	GA (U/g latex)
1	*Euphorbia nivulia*	6.10 ± 0.14	9.20 ± 0.08	465.5 ± 0.37	7.34 ± 0.72
2	*Pedilanthus tithymaloides*	13.2 ± 0.24	52.4 ± 0.05	29.9 ± 0.20	8.65 ± 0.87
3	*Synadenium grantii*	3.53 ± 0.12	9.77 ± 0.40	39.9 ± 0.33	4.04 ± 0.36

Values are expressed as mean ± SD, $n=6$ experiment; PA: protease assay, GA: gelatinase assay, and MCA: milk clotting assay.

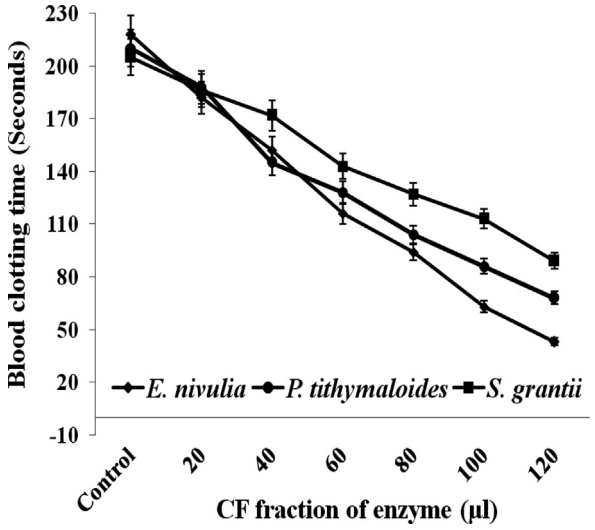

Fig. 1. Coagulation time variation depending on the dose of latex protease (human blood sample).

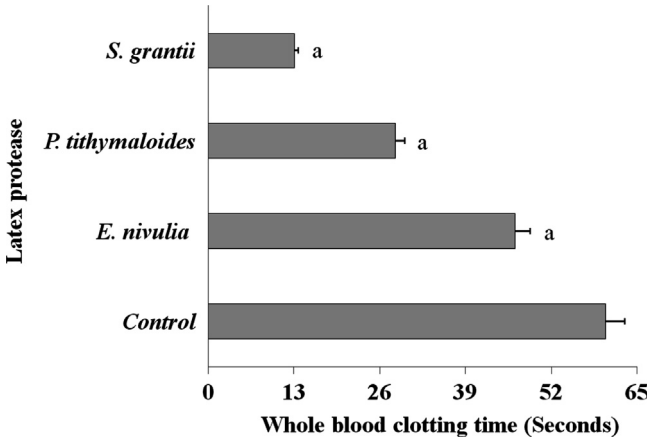

Fig. 2. Coagulation time variation depending on the different latex protease (mice blood sample); $^{a}P<0.05$ compared to control.

The reference range for whole blood clotting time is about 3–5 min at 37 °C. Plant latex proteases of some members of *Euphorbiaceae* viz., *Euphorbia hirta* and *Euphorbia milii* exhibits extremely less blood clotting activity (Badgujar, 2011) whereas *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii* shows remarkable blood clotting activity. The data presented in Fig. 1 illustrates the average of reduced blood clotting time of human blood by the treatment of plant latex protease of *Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii*. Also whole blood clotting time of mice blood sample was reduced by the treatment of these proteases (Fig. 2) and this difference is statistically significant ($P<0.05$). The whole blood clotting time of different mammals viz., *Capra hircus*, *Bos indicus*, *Bubalus bubalis* and *Ovibos moschatus* without adding experimental CF fraction of latex were recorded as 200.17, 132.00, 95.83 and 161.63 s respectively. This blood clotting time was significantly reduced up to 121.50, 31.33, 18.37 and 4.24 s by the treatment of latex protease (CF fraction) of *Pedilanthus tithymaloides* (Table 3). This difference in whole blood clotting time is statistically significant ($P<0.001$).

In order to identify the classes of protease enzymes of plant latices (*Euphorbia nivulia*, *Pedilanthus tithymaloides* and *Synadenium grantii*), the effect of different protease inhibitors have been evaluated on different protease enzyme activities namely protease, gelatinase, milk clotting and blood clotting assay. Table 4 shows the residual activity of the protease enzymes of latices after its inhibition with the following class-specific inhibitors mercuric chloride and iodoacetic acid (inhibitors of cysteine proteases), PMSF (inhibitor of serine proteases), EDTA and phenanthroline (inhibitors of metalloproteases) and pepstatin A (inhibitor of aspartic proteases) using casein (protease assay), gelatin (gelatinase assay), milk

powder (milk clotting assay) and blood sample (blood clotting assay) as substrates.

Maximum inhibition (88.35–97.25 per cent inhibition) of various proteolytic activities like protease (90.52%), gelatinase (89.47%), milk clotting (88.35%) and blood clotting assay (97.25%) of plant latex containing enzyme of *Euphorbia nivulia* occurs in presence of cysteine protease inhibitors namely mercuric chloride and iodoacetic acid (Table 4). Enzymes did not show any significant inhibition in the presence of PMSF and pepstatin-A. Metalloprotease inhibitors like phenanthroline and EDTA showed no significant effect on the activity of latex enzymes ruling out the possibly of the protease enzyme of *Euphorbia nivulia* being a metalloprotein. These results confirm that the enzyme present in the latex of *Euphorbia nivulia* belongs to cysteine protease family.

The proteolytic activities viz., protease, gelatinase, milk clotting and blood clotting assay of the enzyme of *Pedilanthus tithymaloides* latex were inhibited by different class specific protease inhibitors (e.g., cysteine, serine, aspartic, metallo-protease) up to a certain extent but not completely by any class of specific inhibitors (Table 4). Overall inhibition profile of this enzyme exhibits only partial inhibition of activity by different classes of protease inhibitors. Thus, it is difficult to conclude the class of protease of this latex as compared with known proteases from other latex bearing plants.

The effects of various compounds on the enzymatic activity of *Synadenium grantii* latex protease for casein, gelatin, milk powder and blood sample are shown in Table 4. Almost all enzyme activity was lost on incubation with PMSF (inhibitor of serine proteases) for 60 min at 37 °C. As shown in Table 4, typical inhibitors against aspartic and metallo-proteases had no effect on the enzyme activity, indicating that the protease was neither an aspartic nor a metallo-protease. However, the inhibitors for cysteine protease ($HgCl_2$ and IAA) inhibited the enzyme from 11.69% to 55.13%. The strongest inhibition (88.63% to 95.61%) was observed with PMSF. Inhibition of the enzyme activities with PMSF suggests the enzymatic activity of *Synadenium grantii* latex protease belongs to the serine protease category.

Table 5 summarizes the results obtained on phytochemical investigation of CF fraction of plant latices. CF fraction of *Euphorbia nivulia* gave positive test for alkaloids, cynogenic glycosides, phenolics, tannins and saponins. Qualitative analyses of CF fraction of *Pedilanthus tithymaloides* revealed the presence of alkaloids, flavonoids and phenolics but the absence of glycosides, terpenoids, saponins, and tannins. On the other hand, CF fraction of *Synadenium grantii* gave positive test for alkaloids, cynogenic glycosides, phenolics and saponins.

Table 3
Effect of *Pedilanthus tithymaloides* latex protease on whole blood clotting time.

Source of blood	Blood clotting time (s)	
	Control	With latex
Capra hircus	200.17 ± 0.75	121.50 ± 0.84[a]
Bos indicus	132.00 ± 0.89	31.33 ± 1.21[a]
Bubalus bubalis	95.83 ± 0.98	18.37 ± 0.82[a]
Ovibos moschatus	161.63 ± 0.75	04.24 ± 0.82[a]

Values are expressed as mean ± SD, $n=6$ animals in each group.
[a] $P<0.001$ when compared to control.

Table 5
Phytochemical investigation of CF fraction of plant latices.

Sr. no.	Qualitative test	Phytochemical result of CF fraction of latex[a]		
		Euphorbia nivulia	*Pedilanthus tithymaloides*	*Synadenium grantii*
1	Alkaloids	+	+	+
2	Cynogenic glycosides	+	−	+
3	Phenolics	+	+	+
4	Flavonoids	−	+	−
5	Terpenoids	−	−	−
6	Tannins	+	−	−
7	Saponins	+	+	+

[a] Phytochemical tests: − negative and + positive.

Table 6
Effect of latex protease on bleeding time of mice.

Enzyme extract	Protein content (μg)	Bleeding time (s)
Euphorbia nivulia	50	44.64 ± 1.20[a,b]
	100	26.83 ± 1.16[a,b]
Pedilanthus tithymaloides	50	35.14 ± 2.26[a,b]
	100	14.68 ± 0.22[a,b]
Synadenium grantii	50	48.19 ± 2.34[a,b]
	100	30.38 ± 1.64[a,b]
Normal saline	–	59.28 ± 0.58
Control	–	57.17 ± 0.98

Values are expressed as mean ± SD, $n=6$ animals in each group.
[a,b] $P<0.05$ compared to control and normal saline respectively.

Table 4
Inhibition profile of proteolytic activities of laticiferous plants.

Type of inhibitor	Inhibitors[a]	Residual activity (%)											
		Euphorbia nivulia				*Pedilanthus tithymaloides*				*Synadenium grantii*			
		PA	GA	MCA	BCA	PA	GA	MCA	BCA	PA	GA	MCA	BCA
	Control[b]	100	100	100	100	100	100	100	100	100	100	100	100
Cysteine protease	Iodoacetic acid^{+}	15.46	12.46	NT	NT	85.17	88.34	NT	NT	88.56	95.73	NT	NT
	Mercuric chloride^{+}	9.48	10.53	11.65	2.75	88.67	91.27	82.16	39.52	84.47	88.31	56.76	44.87
Serine protease	PMSF^{+}	76.05	83.67	43.87	64.22	84.65	87.13	78.45	41.90	11.37	9.38	9.12	4.39
Metalloprotease	EDTA^{+}	76.63	97.45	NT	NT	90.59	93.56	NT	NT	93.29	78.11	NT	NT
	Phenanthroline^{+}	87.92	84.31	66.95	45.87	84.76	96.32	87.51	46.19	94.65	87.46	63.26	43.41
Aspartic protease	Pepstatin A^{++}	72.49	78.32	42.89	55.96	96.84	92.18	86.22	36.19	97.64	93.15	72.34	35.60

[a] Enzyme was incubated with inhibitors at 37 °C for 60 min and residual activity was measured using casein and gelatin as substrate.
[b] The enzyme activity towards without (W/O) inhibitor was taken as 100%; $^{+}$Concentration of inhibitors: 5 mM; $^{++}$Concentration of inhibitor: 0.1 mM; PA: protease assay; GA: gelatinase assay; MCA: milk clotting assay and BCA: blood clotting assay; NT: not tested.

Significant reduction in bleeding time of albino mice was reported by the treatment of plant latex protease. Briefly, bleeding time of mice is about 57.17 s. It was significantly reduced up to 14.68 s by the treatment of plant latex protease. The reduced whole blood clotting and bleeding times recorded in the experiment with enzyme as compared to those without latex enzyme was evidenced that the plant latices possess coagulant principle. The enzyme fractions significantly ($P < 0.05$) reduced bleeding time in mice as compared to positive (normal saline) and negative control group of animals i.e. mice. On coagulation of whole blood, the protease fractions decreased the coagulation time of whole blood of mice in a dose-dependent manner. In both tests, the magnitude of activity is of the order: *Pedilanthus tithymaloides* > *Euphorbia nivulia* > *Synadenium grantii* (Table 6). At the end of the first, 24, 48, 72 h, 7 days and 14 days after treatment, mice of the test group and those of the control groups revealed no skin lesion. The irritation score obtained was around zero and CF fractions of plant latices of *Pedilanthus tithymaloides*, *Euphorbia nivulia* and *Synadenium grantii* belong to a non-irritating category substance. The results indicate that CF fraction of *Pedilanthus tithymaloides* latex exhibits significant hemostatic activity. It warrants further study for the confirmation of hemostatic agent either protease enzyme of plant latex or specific phytoconstituents (secondary metabolites) of latex.

4. Discussion

The results of inhibition profile of *Euphorbia nivulia* confirm that the enzyme present in the latex belongs to cysteine protease family. Very recently, similar results of enzyme inhibition profile of cysteine protease of *Triticum aestivum* (Fahmy et al., 2004) and *Curcuma longa* (Nagarathnam et al., 2010) by iodoacetic acid are reported. Present work is very well close with the inhibition profile of procerain B, cysteine protease of *Calotropis procera* latex (Singh et al., 2010). The above-mentioned inhibition profile of *Synadenium grantii* protease classified as a member of the serine protease class, with a cysteine residue near its active site. Strong inhibition by PMSF was also reported for some plant serine proteases, such as cucumisin-like protease from latex of *Euphorbia supina* (Arima et al., 2000), subtilisin-like protease from *Cucumis trigonus* (Ullah et al., 2006), and milin of *Euphorbia milii* (Yadav et al., 2006).

Evaluation of the potentiality of *Pedilanthus tithymaloides* showed that the plant latex fraction has hemostatic properties. The latex fraction arrested bleeding from fresh wounds by reducing bleeding and whole blood coagulation time which are important indices of hemostatic activity. These results are good in agreement with the earlier observations of hemostatic activity of stem latex of another member of *Euphorbiaceae* family i.e. *Jatropha gossypifolia* (Oduola et al., 2007). Also, *Pedilanthus tithymaloides* possesses proteolytic enzyme (Bhowmick et al., 2008), thus, this property may be capable of procoagulant activity. The reduction in coagulation time of whole blood by the latex phytoconstituents (protease or other secondary metabolites) indicates that the phytoconstituents may also interfere with the blood coagulation pathways. Thus, these plant latices act as a promising hemostatic agent and it is worth to evaluate it further in detail. However, which principle could affect the clotting property remains uncertain. Purification and characterization of active ingredient (either protein/secondary metabolite) factor responsible for a hemostatic activity is needed.

In conclusion, there is no doubt that, the above mentioned plant latices have procoagulant properties. However, the present investigation needs toxicity studies used in elimination of any harmful side effects before it can be safely recommended for treatment.

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